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REMARKS

The present invention is directed to immunogenic compositions comprising fibroblast growth factor ("FGF") and/or vascular endothelial growth factor ("VEGF"), to methods for treating cancer or hyperproliferative disorders in humans or animals, and also to methods for treating humans or animals in need of an immune response to a growth factor.

Claims 5-13, 15, 17-23 and 25-29 are currently pending in the above-identified application. In response to the Office Action dated April 22, 2002 and in order to facilitate prosecution, Claims 5, 15 and 25 are herein amended, and Claim 30 has been added. No new matter has been added and support for the claims is found in the specification. Applicants submit the following remarks in an effort to address the rejections raised in the Office Action.

1. Rejection of Claims 15 and 17-23 under 35 U.S.C. § 112, first paragraph

In the Office Action dated April 22, 2002, Claims 15 and 17-23 were rejected under 35 U.S.C. § 112, first paragraph, as failing to convey to one of skill in the art that the applicants had the invention at the time the application was filed. The claims remained rejected for reasons of record.

Specifically, the Office Action indicated that the applicants had not shown through any adequate written description or disclosure in the specification that the peptides encoded by the claimed SEQ ID NOS. were structurally related to a receptor binding domain of VEGF. Applicants respectfully traverse this rejection and request reconsideration and withdrawal of the rejection in light of the following remarks.

It is respectfully submitted that one of skill in the art at the time the application was filed would have known that the peptides encoded by SEQ ID NOS. 1-9 were structurally related to the receptor binding domains of VEGF. As evidenced by Neufeld et al. the binding domains of VEGF were well known in the art at the time of the application. (Neufeld et al. *The FASEB Journal*, 13:9-22 (January, 1999), provided herewith as Attachment A; *see also* Ortega et al. *Frontiers in BioScience* 4, d141-152 (February, 1999), provided herewith as Attachment B). Applicants specifically describe in the disclosure the amino acid sequences that are claimed in

SEQ ID NOS 1-9 (see page 31, lines 20-30 and page 32, lines 1-10). As such, one of ordinary skill in the art would recognize the sequences as receptor binding regions of VEGF.

In light of the above remarks and enclosed references, applicants believe that Claims 15 and 17-23 are in condition for allowance and respectfully request reconsideration and withdrawal of this rejection.

Claim 30 has been added herein in order to facilitate prosecution. Claim 30 is specifically directed to an immunogenic peptide consisting of that portion of vascular endothelial growth factor receptor binding domain as represented by SEQ ID NO: 6. Support for the Claim can be found throughout the specification and specifically in Example 15 on page 61, lines 19-20 which names SEQ ID NO: 6 as belonging to the receptor binding domain of VEGF.

In light of the above described amendments and remarks, applicants believe that the claims are in condition for allowance and respectfully request reconsideration and withdrawal of this rejection.

2. Rejection of Claim 6 under 35 U.S.C. § 102(b)

In the Office Action dated April 22, 2002, Claim 6 was rejected under 35 U.S.C. § 102(b) as being anticipated by Senoo et al. (EP281822). The Office Action indicated that Senoo et al. describes a “peptide that is identical to that disclosed in SEQ ID NO: 1.” Specifically, the Office Action indicated that the claim was anticipated because “inherently, the properties discovered by applicant in the instant application can be found in the peptide discovered by Senoo et al. (see sequence alignment).” Applicants respectfully traverse this rejection and request reconsideration and withdrawal of the rejection in light of the following remarks.

Applicants have discovered a novel and previously unidentified genus of peptides from the heparin binding domain regions of FGF that are immunogenic. Applicants’ invention comprises compositions that are characterized by the heparin binding domain of FGF and their immunogenicity. Specifically, the phrasing of Claim 6 uses closed language, “consists of”, indicating that only the amino acid sequence disclosed in the specification (page 31, lines 20-22) is claimed. A claim in a patent application is anticipated under 35 U.S.C. § 102(b) only when each and every element of the claim is disclosed by single piece of prior art. *See Glaxco Inc. v.*

Novopharm Ltd., 52 F.3d 1043 (Fed. Cir. 1995) and unlike the composition described in Claim 6, Senoo et al. fail to identify immunogenic peptide fragments located in the heparin binding domain of FGF.

Senoo et al. describe the cloning and expression of FGF muteins for the purpose of stimulating angiogenic activity. The peptides described by Senoo et al. comprise amino acid sequences longer than that described in Claim 6 and are neither confined to the heparin binding domain nor described relative to the heparin binding domain. Furthermore, Senoo et al fail to identify FGF sequences having immunogenic properties. Therefore, because Senoo et al. do not describe each and every element of Claim 6, they fail to anticipate applicants' invention.

The Office Action indicated that applicants' discovery was inherent in the peptide described in Senoo et al. However, inherency may not be established by probabilities or possibilities. *See In re Oelrich and Divigard*, 212 U.S.P.Q. 323 (C.C.P.A. 1981). It is possible that the peptide discussed by Senoo et al. may stimulate angiogenic activity from a binding domain of FGF similar to that claimed by applicants, but it is also possible that a response described by Senoo et al. came from an undiscovered or unidentified binding domain of the disclosed peptide sequence. It is the immunogenic response from the exact heparin binding domain of FGF, which Senoo et al fail to describe, that applicants claim in Claim 6 of the application. The mere fact that a certain thing may result from a given set of circumstances is not sufficient. *See Id.* Therefore, applicants respectfully submit that the reliance on the doctrine of inherency is improper in this case.

In light of the above remarks, applicants believe that Claim 6 is in condition for allowance and respectfully request reconsideration and withdrawal of this rejection.

3. *Rejection of Claims 5-13, 15, 17-23 and 25-29 under 35 U.S.C. § 112, second paragraph*

In the Office Action dated April 22, 2002, Claims 5-13, 15, 17-23 and 25-29 were newly rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim subject matter which the applicants regard as the invention. Specifically, the Office Action indicated that in Claims 5, 15 and 25 the phrase "immunogenic

fragments” was unclear regarding “whether immunogenic fragments of FGF or VEGF are to contain heparin binding domains or receptor binding domains, respectively.”

Per Examiner’s request, Claims 5, 15 and 25 are herein amended to particularly point out and distinctly claim subject matter which applicants regard as their invention. Applicants have replaced the word “thereof” with specific language found in the claim itself. Support for the amendment can be found throughout the specification. Specifically, support can be found on page 31, lines 10-15.

Claims 6-13 are dependant claims of Claim 5 and, in light of the above discussed amendment to the Claim, distinctly claim subject matter which applicants regard as their invention.

Claims 17-23 are dependant claims of Claim 15 and, in light of the above discussed amendment to the Claim, distinctly claim subject matter which applicants regard as their invention.

Claims 26-29 are dependant claims of Claim 25 and, in light of the above discussed amendment to the Claim, distinctly claim subject matter which applicants regard as their invention.

The present Claims particularly point out and distinctly claim the subject matter which applicants regard as the invention and are believed to be in condition for allowance. Therefore, applicants respectfully request reconsideration and withdrawal of the rejection.

4. Rejection of Claims 5, 7-10 and 25-29 under the judicially created doctrine of obviousness-type double patenting

Pending Claims 5, 7-10 and 25-29 were newly rejected under the judicially created doctrine of obviousness-type double patenting as being “unpatentable over claims 1-3, 6-8, 15-17, and 19-21 of U.S. Patent No. 5,919,459 (Nacy et al.).”

The Office Action indicated that Claims 5, 7-10 and 25-29, drawn to immunogenic compositions comprising peptide fragment FGF (heparin binding domain) or FGF (heparin binding domain) and VEGF (receptor binding domain), were similar to Claims 1-3, 6-8, 15-17 and 19-21 of Nacy. Specifically, the Office Action indicated that the Claims in Nacy et al. were

“drawn to similar compositions, with the same limitations set forth in the instantly claimed invention.”


The Office Action also indicated that Claims 25-29 of applicants' invention “are drawn to a combination of FGF and VEGF peptides, which would have been an obvious combination to make because Claims 1-3, 6-8, 15-17 and 19-21 of [Nacy] have already set forth the basic elements of instantly claimed invention.”

With respect to Examiner's rejection, applicants submit that this rejection is not necessary to further consideration of the claims and request it be held in abeyance until allowable subject matter is indicated by the Examiner. *See* 37 C.F.R. § 1.111(b). Once allowable subject matter is indicated, applicants will fully respond to this rejection and file a terminal disclaimer where applicable.

5. *Conclusion*

In light of the amendments, Applicants are of the opinion that Claims 5-13, 15, 17-23 and 25-29 are now in condition for allowance. Such action is respectfully requested. If the Examiner believes any informalities remain in the application which may be corrected by Examiner's Amendment, or there are any other issues which can be resolved by telephone interview, a telephone call to the undersigned attorney at (404) 745-2463 is respectfully solicited.

Respectfully submitted,


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Vascular endothelial growth factor (VEGF) and its receptors

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ABSTRACT Vascular endothelial growth factor (VEGF) is a highly specific mitogen for vascular endothelial cells. Five VEGF isoforms are generated as a result of alternative splicing from a single VEGF gene. These isoforms differ in their molecular mass and in biological properties such as their ability to bind to cell-surface heparan-sulfate proteoglycans. The expression of VEGF is potentiated in response to hypoxia, by activated oncogenes, and by a variety of cytokines. VEGF induces endothelial cell proliferation, promotes cell migration, and inhibits apoptosis. *In vivo* VEGF induces angiogenesis as well as permeabilization of blood vessels, and plays a central role in the regulation of vasculogenesis. Deregulated VEGF expression contributes to the development of solid tumors by promoting tumor angiogenesis and to the etiology of several additional diseases that are characterized by abnormal angiogenesis. Consequently, inhibition of VEGF signaling abrogates the development of a wide variety of tumors. The various VEGF forms bind to two tyrosine-kinase receptors, VEGFR-1 (*flt-1*) and VEGFR-2 (*KDR/flk-1*), which are expressed almost exclusively in endothelial cells. Endothelial cells express in addition the neuropilin-1 and neuropilin-2 coreceptors, which bind selectively to the 165 amino acid form of VEGF (VEGF₁₆₅). This review focuses on recent developments that have widened considerably our understanding of the mechanisms that control VEGF production and VEGF signal transduction and on recent studies that have shed light on the mechanisms by which VEGF regulates angiogenesis.—Neufeld, G., Cohen, T., Gengrinovitch, S., Poltorak, Z. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J.* 13, 9–22 (1999)

Key Words: hypoxia · angiogenesis · oncogene · heparan-sulfate proteoglycan · cytokine

OVERVIEW OF EARLY STUDIES

Vascular endothelial growth factor (VEGF)² had been characterized as a heparin binding angiogenic growth factor displaying high specificity for endothe-

lial cells (1, 2). Vascular permeability factor (VPF) had been characterized as a protein that promotes extravasation of proteins from tumor-associated blood vessels (3). It was subsequently realized that the permeability-inducing factor and the endothelial cell growth factor are encoded by a single VEGF gene, and that several VEGF isoforms are produced from this gene by alternative splicing to form active disulfide-linked homodimers (4–6).

These initial discoveries were followed by the identification of specific VEGF receptors, first at the level of binding/cross-linking studies (7, 8) and then by identification of the VEGFR-1 (*flt-1*) and VEGFR-2 (*KDR/flk-1*) genes that encode VEGF specific tyrosine-kinase receptors. These receptors are characterized by the presence of seven immunoglobulin-like domains in their extracellular parts and can therefore be regarded as a new subfamily of tyrosine-kinase receptors (9, 10) (Fig. 1). Important advances were also made regarding the biological role of VEGF. It was discovered that VEGF is expressed in spatial and temporal association with physiological events of angiogenesis *in vivo* (11, 12). Inhibition of VEGF activity by neutralizing antibodies or by the introduction of dominant negative VEGF receptors into endothelial cells of tumor-associated blood vessels resulted in the inhibition of tumor growth and in tumor regression, indicating that VEGF is a major initiator of tumor angiogenesis (13, 14). Furthermore, it was found that VEGF expression is potentiated by hypoxia and that the potentiation of VEGF production in hypoxic areas of solid tumors contributes significantly to VEGF-driven tumor angiogenesis (15, 16). VEGF-induced angiogenesis was also found to play an important role in the etiology of several additional diseases associated with abnormal angiogenesis (17, 18) and in wound repair (19).

These advances were accompanied by the identification of additional growth factors belonging to the

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² Abbreviations: bFGF, basic fibroblast growth factor; HIF-1, hypoxia-inducible factor 1; IL, interleukin; IRES, internal ribosomal entry site; PDGF, platelet-derived growth factor; PlGF, placenta growth factor; KGF, keratinocyte growth factor; TGF, transforming growth factor; UTR, untranslated region; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; vHL, von Hippel-Landau; VPF, vascular permeability factor.

formation in homozygous animals. Embryos lacking the VEGFR-2 gene die before birth because differentiation of endothelial cells does not take place and blood vessels do not form (28). A recent study has indicated that VEGFR-2 is required for the differentiation of endothelial cells and for the movement of primitive precursors of endothelial cells from the posterior primitive streak to the yolk sac, a precondition for the subsequent formation of blood vessels (30). Disruption of the gene encoding the VEGFR-1 receptor did not prevent the differentiation of endothelial cells in homozygous animals, but the development of functional blood vessels from these endothelial cells was severely impaired (29). Activation of the VEGFR-1 receptor promotes cell migration but does not seem to induce cell proliferation efficiently (31, 32). It is thus possible that decreased cell migration or defects in endothelial cell-cell or cell-matrix interactions result in the defective organization of blood vessels in mice lacking functional VEGFR-1 (29).

Hypoxia-driven retinal angiogenesis as a model for the role of angiogenesis during organ development

Hypoxia-induced VEGF production may provide the driving force that stimulates the angiogenesis that accompanies organ formation during development. Perhaps the best-studied example of such an event is the development of the retina and the associated network of retinal blood vessels. The picture that has emerged from the retina studies may also be true for the development of vascular systems in other organs. As the retina develops, astrocytes and neuronal precursors spread out and migrate away from the existing blood vessels. As the distance between the astrocytes and the existing vasculature increases, the astrocytes encounter progressively increasing hypoxic conditions. The astrocytes are more sensitive to hypoxia than the comigrating neuronal cells, and thus serve as hypoxia sensors. The hypoxia stimulates the astrocytes to produce VEGF, and the VEGF sets in motion an angiogenic response (33, 34). A VEGF concentration gradient is formed that stimulates the growth of new blood vessels toward the VEGF-producing astrocytes. The result is that growing blood vessels follow the astrocytes that continue to migrate outward. As the hypoxic pressures ease after the arrival of the blood vessels, the production of VEGF by the astrocytes decreases. However, a certain threshold concentration of VEGF is required to inhibit apoptosis of the endothelial cells and is essential for the stabilization of the newly formed blood vessels. The falling levels of VEGF cause disassembly of some of these vessels, leaving intact a vascular network that is finely tuned to respond to the demands of the organ (35). The remaining vessels are later stabilized and rendered insensitive to VEGF withdrawal by pericytes

that follow in the wake of the endothelial cells to cover the newly formed blood vessels (36).

Deregulated VEGF production results in angiogenic diseases of the retina

When cells lose the ability to control the synthesis of VEGF for any number of reasons, angiogenic disease ensues. In retinopathy of prematurity babies are placed in hyperoxygenated incubators because their lungs are not yet fully developed. This causes the astrocytes that are the sensors of hypoxia during the development of the retina to decrease VEGF production, causing newly formed blood vessels to regress and halting the orderly process of retinal angiogenesis during retina development. When the babies are moved out of the incubators, all the cells in the retina suddenly experience extreme hypoxia and produce simultaneously large amounts of VEGF. This overproduction results in rampant angiogenesis, which is not as finely choreographed as the angiogenesis that takes place during normal retinal development. The ensuing deregulated growth of blood vessels is the cause of blindness in babies that have been subjected to such treatment (37, 38). A similar sequence of events can also be seen in other types of retinopathies in which partial or general ischemia of the retina is accompanied by overexpression of VEGF and hyperproliferation of blood vessels leading to blindness (17, 18, 39, 40). This model is also supported by recent experiments that have demonstrated hyperproliferation of blood vessels in transgenic mice engineered to overproduce VEGF in retinal cells (41).

When tissues are exposed to high concentrations of VEGF, additional abnormalities besides the hyperproliferation of blood vessels can be observed. Newly formed blood vessels of quail embryos exposed to high concentrations of VEGF undergo unregulated and excessive fusion of vessels that results in the formation of vessels with abnormally large lumens. This unregulated fusion leads in extreme cases to the formation of fused vascular sacs that obliterate the identity of individual vessels (42). If, in contrast, the VEGF supply is reduced or completely inhibited, the result is impaired angiogenesis, which can lead to the inhibition of organ development. A truncated soluble VEGFR-1 was used to inhibit angiogenesis during the development of the corpus luteum in a rat model of hormonally induced ovulation. The inhibition of angiogenesis resulted in the complete inhibition of corpus luteum development (43). These experiments support the hypothesis that the availability of angiogenic factors such as VEGF may be a major limiting factor that controls the extent of organ development and growth (44).

The role of VEGF in tumor angiogenesis

Even though tumors are composed of dedifferentiated cells and do not exhibit an organized structure,

from the point of view of angiogenesis a growing tumor may be viewed as a developing new organ. Angiogenesis is as essential for the growing tumor as it is for a developing organ such as the corpus luteum, since the delivery of blood-borne nutrients to the tumor cells is essential for their survival (43, 45). Therefore the production of angiogenic factors by tumorigenic cells is essential for the development of solid tumors (46). Initial studies have revealed that when VEGF signaling is inhibited, tumor angiogenesis and, consequently, tumor growth are impaired (13, 14). VEGF also contributes to the development of tumors because of its ability to induce permeabilization of blood vessels. VEGF induces the formation of fenestrations in blood vessels (47, 48) and the formation of vesiculo-vacuolar organelles that form channels through which blood-borne proteins can extravasate (49). This leads to the formation of an extravascular fibrin gel, which provides a matrix that supports the growth of endothelial cells and tumor cells and allows invasion of stromal cells into the developing tumor (50).

In many types of tumors, elevated levels of VEGF production can often be detected in tumor cells located at the extreme periphery of the tumor where there is no hypoxia. It was subsequently observed that activated oncogenes that are part of the *ras*/MAP-kinase signal transduction pathway potentiate VEGF mRNA expression (51, 52). Hypoxia-independent production of VEGF by tumorigenic cells can also be brought about by the inactivation of tumor suppressors such as p53 (53, 54) or by exogenous factors such as hormones or growth factors (55). The molecular mechanisms by which these inducers affect VEGF production in normal and tumor cells are discussed in greater detail in the section dealing with the regulation of VEGF production.

As tumors expand, the cells within the expanding mass of the tumor are frequently deprived of oxygen because their distance from the nearest blood vessels increases. This results in the generation of hypoxic regions within tumors; the tumorigenic cells within these hypoxic areas respond by the stimulation of VEGF production, which then triggers angiogenesis using mechanisms similar to the mechanisms by which astrocytes sense hypoxia and induce angiogenesis in the developing retina. This results in particularly high levels of VEGF expression in hypoxic regions, which are usually located near necrotic areas within tumors (56). The generality of this mechanism of VEGF induction explains why VEGF seems to be involved in the induction of tumor angiogenesis in so many types of diverse tumors. Another VEGF-related mechanism by which tumor angiogenesis can be induced involves production of VEGF-like proteins by viruses. The AIDS virus protein, HIV-1/Tat, can bind and activate VEGFR-2 (57). This probably represents a mechanism by which the AIDS virus en-

hances the angiogenic stimuli provided by Kaposi sarcoma herpes virus-encoded proteins (58), contributing to the development of Kaposi sarcoma in AIDS patients.

The use of VEGF and VEGF function inhibitors as drugs for various diseases associated with angiogenic disorders

The initial studies indicating that inhibition of VEGF signal transduction can inhibit tumor progression (13, 14) were followed by studies that have indicated that inhibition of VEGF signaling inhibits the development of many types of tumors (59). Recent studies also suggest that inhibition of VEGF function abrogates tumor metastasis, possibly because the tumor cells come into contact with a lesser concentration of blood vessels (60). These observations have sparked intense efforts directed at the development of efficient inhibitors of VEGF production and VEGF signal transduction for anti-tumorigenic purposes. Efforts to inhibit VEGF-induced tumor angiogenesis include the development of humanized neutralizing anti-VEGF monoclonal antibodies (61), inhibitory soluble VEGF receptors (43, 62, 63), antisense VEGF mRNA expressing constructs (64, 65), VEGF-toxin conjugates (66), antagonistic VEGF mutants (67), and inhibitors of VEGF receptor function (60, 68, 69). All these strategies hold promise, and it remains to be seen whether one of these approaches will lead to the development of an efficient inhibitor of VEGF-induced tumor angiogenesis.

The angiogenic properties of VEGF have also been exploited recently to induce *in vivo* angiogenesis for the treatment of two types of diseases associated with impaired blood supply. In some cases, VEGF was delivered to the sites of interest as a protein (70). In other cases expression plasmids containing the VEGF cDNA or recombinant viruses were used in a gene therapy approach (71–73). Successful attempts aimed at induction of collateral blood vessels in ischemic heart disease, critical limb ischemia, and reendothelialization by VEGF have been reported recently (70, 74–76), and efforts are continuing.

THE REGULATION OF VEGF PRODUCTION

The regulation of VEGF production by growth factors, cytokines, and other extracellular molecules

VEGF is a key regulator of angiogenesis, and its expression in producing cells is regulated by a plethora of external factors. Cytokines, growth factors, and gonadotropins that do not stimulate angiogenesis directly can modulate angiogenesis by modulating VEGF expression in specific cell types, and thus exert an indirect angiogenic or anti-angiogenic effect. Fac-

tors that can potentiate VEGF production include fibroblast growth factor 4 (FGF-4) (77), PDGF (78), tumor necrosis factor α (79), transforming growth factor β (TGF- β) (80), keratinocyte growth factor (KGF) (81), IGF-I (82), interleukin 1 β (IL-1 β) (83), and IL-6 (84). Other cytokines such as IL-10 and IL-13 can inhibit the release of VEGF (85). An interesting example illustrating the complexity of the regulation of VEGF production during a biological process is the regulation of VEGF production during dermal wound healing. The expression of KGF is strongly potentiated during wound repair (86). KGF, in turn, induces VEGF production in keratinocytes. In addition, hydrogen peroxide, a VEGF-inactivating oxidant (87) produced by neutrophils that invade the wound as part of the healing process, also potentiates VEGF production by keratinocytes (88). The production of VEGF in keratinocytes is also strongly induced by UV-B radiation, again as part of a wound repair mechanism that involves angiogenesis (88). Another small molecule that up-regulates VEGF expression is nitric oxide. Nitric oxide contributes to the blood vessel-permeabilizing effects of VEGF and to VEGF-stimulated vasodilatation (89–91). The production of nitric oxide is in turn up-regulated by VEGF, indicating that a positive feedback loop exists between these two factors (92, 93).

The mechanism by which hypoxia regulates VEGF production

Hypoxia and hypoglycemia are major stimulators of VEGF expression (15). The production of other VEGF family members such as VEGF-B, VEGF-C, and PlGF does not seem to be potentiated by hypoxia even though some of these factors such as VEGF-C are strong angiogenic factors in their own right (94, 95). The mechanisms that regulate VEGF production by oxygen availability have therefore received particular attention in recent years and are slowly being worked out. The mechanisms responsible for induction of VEGF and erythropoietin expression are similar (96). Hypoxia-induced transcription of VEGF mRNA is apparently mediated, at least in part, by the binding of hypoxia-inducible factor 1 (HIF-1) to an HIF-1 binding site located in the VEGF promoter (97, 98). It turns out that some mechanisms that lead to elevated VEGF production independently of hypoxia actually short-circuit the normal hypoxia sensing mechanism that regulates VEGF expression. For example, it was observed that the oncogene *v-src* can induce expression of HIF-1, thereby short-circuiting the HIF-1-dependent hypoxia sensing mechanism and leading to increased expression of VEGF (99). Several additional HIF-1-like factors regulate VEGF production, but these transcription factors are not as well characterized (100, 101). In addition to the induction of transcription, hypoxia promotes the sta-

bilization of the VEGF mRNA by proteins that bind to sequences located in the 3' untranslated region (UTR) of the VEGF mRNA (102–104). Recently, one such protein has been identified as the HuR mRNA binding protein (105). It is clear that additional proteins stabilize the VEGF mRNA, but their identity is still unknown.

Recent evidence indicates that the hypoxia-mediated elevation in VEGF transcription is also mediated by sites that are found in the 5' untranslated region of the VEGF mRNA (UTR), which is particularly long. The 5' UTR contains an alternative transcription initiation site and an active internal ribosomal entry site (IRES) located downstream of this alternative initiation site. MRNA initiated from this alternative start site contains the IRES. The translation of such uncapped mRNA is probably regulated primarily by the IRES and may be advantageous under conditions in which cap-dependent translation is inhibited, as is the case under stressful conditions such as the conditions prevailing during hypoxia (106, 107).

Regulation of VEGF expression by the von Hippel-Landau (vHL) and p53 genes

Inactivation of tumor suppressors is an additional mechanism that leads to overexpression of VEGF in tumor cells. Cerebellar hemangioblastomas and human renal carcinomas are highly vascular, nonnecrotic, and presumably nonhypoxic tumors producing high levels of VEGF. Cells derived from such tumors contain mutations in the gene encoding the vHL tumor suppressor gene (108). This observation suggests that mutations in the vHL gene are associated with increased angiogenesis, and it was indeed found that cells from such tumors display increased VEGF expression as a result of the inactivation of vHL (109, 110). Wild-type vHL inhibits the production of several hypoxia-regulated proteins such as the GLUT-1 glucose transporter gene in addition to VEGF. vHL inhibition of VEGF expression is mediated by transcriptional and posttranscriptional mechanisms (111, 112). At the posttranscriptional level, vHL inhibits the activity of protein kinase C zeta and delta (113). In the absence of wild-type vHL, these kinases are active and the VEGF mRNA is stabilized as a result of the constitutive interaction of several proteins that are normally induced by hypoxia with a 500 base region in the 3' UTR (54). At the transcriptional level, vHL forms a complex with the Sp1 transcription factor and inhibits SP1-mediated VEGF expression as a result of the binding of SP1 to a specific region in the VEGF promoter (111). These Sp1 binding sites are also important for PDGF-induced VEGF expression; mutations that inhibit Sp1 binding also abolish PDGF-induced VEGF expression (78). The expression of PDGF-BB is also potentiated by hypoxia (114).

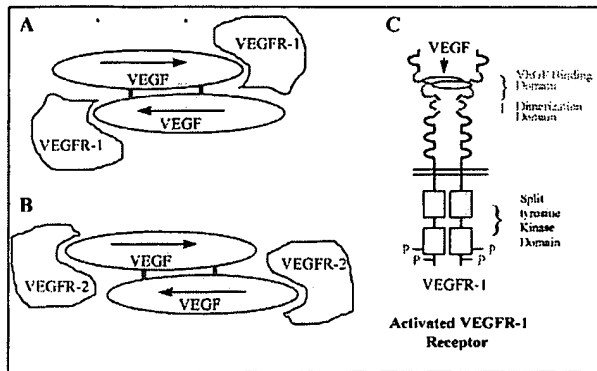


Figure 2. The interaction of VEGF with its signaling tyrosine-kinase receptors. The interaction of VEGF with the binding sites of VEGFR-1 (A) and VEGFR-2 (B) are shown as viewed from above. The two VEGF monomers are shown in a head-to-tail orientation, indicated by arrows, and held together by disulfide bonds shown in orange. The main cluster of VEGF amino acids that bind VEGFR-1 is located at one end of the VEGF monomer (A); the main cluster of VEGF amino acids that bind VEGFR-2 is located at the opposite pole of the VEGF monomer (B). Nevertheless, the VEGF domains that bind VEGFR-1 and VEGFR-2 overlap as shown. The main VEGF binding domain of the VEGFR-1 and VEGFR-2 receptors is located in immunoglobulin-like loop 2, but loop 3 also participates in the binding. The two VEGFR-1 receptors form a dimer that undergoes autophosphorylation on tyrosine residues located in the cytoplasmic part of the VEGFR-1 receptors (P), leading to the initiation of signal transduction. The dimer is held together by the interaction of each VEGFR-1 with a common VEGF dimer and is further stabilized by interactions between amino acids located at the loop 4 dimerization domain (C). This model is based primarily on data derived from refs 155 and 157.

Another tumor suppressor associated with the control of VEGF expression seems to be P53. The loss of the wild-type P53 is associated with increased angiogenesis in developing tumors (115). Wild-type P53 was identified as an inhibitor of VEGF production (116), and mutated P53 was observed to potentiate VEGF expression (53). However, a recent report apparently contradicts these findings and indicates that wild-type P53 may not function as an inhibitor of VEGF expression (117).

THE VEGF SPLICE VARIANTS AND THE ROLE OF CELL-SURFACE HEPARAN-SULFATE PROTEOGLYCAN

Similarities and differences between the VEGF splice forms

Five human VEGF mRNA species encoding VEGF isoforms of 121, 145, 165, 189, and 206 amino acids (VEGF₁₂₁₋₂₀₆) are produced by alternative splicing of the VEGF mRNA (5, 6, 118, 119). An important biological property that distinguishes the different VEGF

isoforms is their heparin and heparan-sulfate binding ability. VEGF₁₂₁ lacks the amino acids encoded by exons 6 and 7 of the VEGF gene (120) and does not bind to heparin or extracellular matrix (121). The addition of the 44 amino acid-long peptide encoded by exon 7 of the VEGF gene distinguishes VEGF₁₆₅ from VEGF₁₂₁ and confers on VEGF₁₆₅ a heparin binding ability (121, 122). VEGF₁₄₅ is distinguished by the presence of the 21 amino acid peptide encoded by exon 6. These amino acids contain a second independent heparin binding domain and also contain elements that enable the binding of VEGF₁₄₅ to the extracellular matrix (119). VEGF₁₈₉ and VEGF₂₀₆ contain the peptides encoded by exons 6 and 7 and display a higher affinity to heparin and heparan-sulfates than VEGF₁₄₅ or VEGF₁₆₅. VEGF₁₈₉ is not secreted into the medium of VEGF₁₈₉-producing cells. It is sequestered on heparan-sulfate proteoglycans of cell surfaces and in the extracellular matrix, and there are indications that it is less active than either VEGF₁₂₁ or VEGF₁₆₅ *in vivo* (123). Proteases such as plasmin can cleave it and release an active soluble proteolytic fragment of 110 amino acids (VEGF₁₁₀) (124, 125). The three secreted VEGF splice forms VEGF₁₂₁, VEGF₁₄₅, and VEGF₁₆₅ induce proliferation of endothelial cells and *in vivo* angiogenesis (119, 121, 126). When expression of the splice variants was examined, it was found that most cell types produce several VEGF variants simultaneously. Usually the 121 and 165 forms were the predominant forms, but expression of the 189 form could also be seen in most VEGF-producing cell types (127). In contrast, VEGF₁₄₅ expression seems to be more restricted, and it was found to be expressed in cells derived from reproductive organs (119, 128, 129).

The interaction between VEGF and cell-surface heparan-sulfates and its role

The heparin binding forms of VEGF can bind to cell-surface and extracellular matrix-associated heparan-sulfate proteoglycans and can release angiogenic factors such as bFGF, which are stored on heparan-sulfates of the extracellular matrix (130). This observation is significant because VEGF and bFGF synergize with respect to their ability to induce angiogenesis (131). Extracellular matrix-associated heparan-sulfate proteoglycans may also function as an extracellular storage place for the heparin binding VEGF isoforms (121). Heparan-sulfate proteoglycans regulate the interaction of several heparin binding growth factors with their respective receptors and, consequently, their biological activity (132). Early experiments have indicated that the binding of ¹²⁵I-VEGF₁₆₅ to endothelial cell-surface receptors such as VEGFR-2 can be strongly enhanced by heparin (133). However, subsequent experiments have shown that heparin-like molecules do not potentiate signif-

icantly the binding of native VEGF₁₆₅ to VEGFR-2. Oxidized VEGF₁₆₅ and VEGF₁₂₁ lose their ability to bind to VEGFR-2. Cell-surface heparan-sulfate proteoglycans such as glypican (134) fulfill a chaperone-like role and can restore the VEGFR-2 binding ability of oxidized VEGF₁₆₅ (S. Gengrinovitch, unpublished results). In contrast, the VEGFR-2 binding ability of VEGF₁₂₁ cannot be restored by heparan-sulfate proteoglycans after oxidative damage (87). This observation may explain why VEGF₁₂₁ was reported to be much less potent than VEGF₁₆₅ by some researchers (135), whereas others report smaller differences in activity (87, 136). It is possible that VEGFs sustain various degrees of damage during their purification from cells or bacteria expressing recombinant VEGF and that such damage can be readily detected in the case of VEGF₁₂₁, because cell-surface heparan-sulfates do not restore the activity of such damaged VEGF₁₂₁.

Several studies indicate that heparan-sulfates may bind to VEGF receptors such as VEGFR-2 and regulate their VEGF₁₆₅ binding ability (137, 138). However, the binding of VEGF₁₂₁ to VEGFR-2 is not affected by heparin or heparan-sulfate proteoglycans, indicating that heparan-sulfates probably cannot modulate the VEGF₁₂₁ binding ability of this particular receptor (87). However, the VEGF₁₂₁ binding ability of other types of VEGF receptors may be directly affected by heparan-sulfates (122).

THE VEGF RECEPTORS

Tyrosine-kinase receptors of VEGF

Two VEGF receptors belonging to the tyrosine-kinase receptor family have been identified and cloned: the VEGFR-1 and the VEGFR-2 receptors (9, 10, 139, 140). Along with the VEGFR-3 receptor, which is expressed in lymph vessels (141) and binds VEGF-C and VEGF-D, these receptors form a subfamily distinguished by the presence of seven immunoglobulin-like loops in their extracellular part and a split tyrosine-kinase domain in their intracellular part (142) (Fig. 1). The VEGFR-2 and VEGFR-1 receptors are expressed predominantly in endothelial cells, but a few additional types of cells express one or both of these receptors. The VEGFR-1 receptor is expressed in trophoblast cells (143), monocytes (31), and renal mesangial cells (144). VEGFR-2, on the other hand, is also expressed in hematopoietic stem cells, megakaryocytes, and retinal progenitor cells (145, 146). In the retinal, two functional VEGFR-2 forms are expressed as a result of alternative splicing (147). In addition, there are tumorigenic cell types that express VEGFR-2 or VEGFR-1. These include, for example, malignant melanoma cells (122, 148, 149). The VEGFR-2 and VEGFR-1 receptors are probably activated by all VEGF isoforms but fulfill somewhat

different functions *in vivo*, as targeted gene disruption experiments revealed (28, 29). Both receptor types can transduce signals of other growth factors belonging to the VEGF family, as shown in Fig. 1, but only the VEGF isoforms can bind to VEGFR-1 and to VEGFR-2. Both receptors are glycosylated; in the case of VEGFR-2, only the final glycosylated form is capable of undergoing autophosphorylation in response to VEGF (150).

The expression of VEGFR-2 and VEGFR-1 was reported to be affected by hypoxia, although to a lesser extent than that of VEGF. The transcription of VEGFR-1, but not that of VEGFR-2, is enhanced by hypoxia (151). VEGFR-2 production is also up-regulated under hypoxic conditions, but the mechanism responsible for the induction seems to be posttranscriptional (152). This hypoxia-induced change in VEGFR-2 and VEGFR-1 expression may be triggered indirectly, since VEGF potentiates the expression of both receptor types (153, 154).

The interaction of VEGF with VEGFR-1 and VEGFR-2

Two separate domains of VEGF interact with VEGFR-2 and VEGFR-1. Alanine-scanning mutagenesis has revealed that Arg(82), Lys(84), and His(86), are critical for the binding of VEGF to VEGFR-2, while Asp(63), Glu(64), and Glu(67) are required for the binding of VEGF to VEGFR-1. These binding domains are located at opposite ends of the VEGF monomer. In the mature VEGF dimer, the monomers are linked in a rough 'head-to-tail' fashion (with a large overlap) by disulfide bridges so that the main VEGFR-2 binding domains are at opposite ends of the molecule, as are the main VEGFR-1 binding domains (Fig. 2A, B) (155, 156). This spatial arrangement is in agreement with observations indicating that mutations within the VEGFR-2 binding site of VEGF have a minimal effect on the binding of VEGF to VEGFR-1, and that mutants affecting the VEGFR-1 binding site of VEGF do not affect the binding of VEGF to VEGFR-2 (155). Furthermore, mutated VEGF deficient in VEGFR-2 binding ability did not induce proliferation of endothelial cells, whereas mutants deficient in their VEGFR-1 binding ability were still able to induce endothelial cell proliferation (155). Nevertheless there is considerable overlap in the binding domains that interact with VEGFR-1 and VEGFR-2 at the groove formed between the VEGF monomers with which both VEGF receptors interact (Fig. 2A, B) (157). The arrangement of the receptor binding sites on VEGF indicates that a VEGF dimer may be able to bind and link together two VEGF receptors to form signaling homo- or heterodimers of receptors (Fig. 2C) (157, 158). Indeed, there is some evidence indicating that VEGFR-2 and VEGFR-1 can form heterodimers after VEGF binding (159). In the

case of VEGFR-1, it was recently determined that the VEGF binding site is located in the second and third immunoglobulin-like loops and that two VEGFR-1 receptors can be linked by a VEGF bridge (157). It was also determined that the fourth immunoglobulin-like loop contains a receptor dimerization domain (157, 160–162) (Fig. 1). The second and the third immunoglobulin-like loops of VEGFR-2 are also sufficient for VEGF binding (158); its fourth immunoglobulin-like loop may also function as a dimerization domain, although there is no experimental data as yet to prove that assumption. The c-kit receptor that belongs to the PDGF subfamily of tyrosine-kinase receptors contains five immunoglobulin-like domains (as compared to seven in VEGF receptors). This receptor also possesses a dimerization domain located in its fourth immunoglobulin-like loop and a ligand binding site in the second and third immunoglobulin-like loops (163). These structural similarities indicate that the VEGF and PDGF tyrosine-kinase receptor subfamilies are evolutionarily linked; this assumption was strengthened by a study that compared the structure of VEGFR-1 with the structure of several receptors belonging to the PDGF receptor family (164).

Biological responses mediated by the activation of VEGFR-1 and VEGFR-2

Activation of the VEGFR-2 receptor by VEGF in cells devoid of VEGFR-1 results in a mitogenic response, while the activation of VEGFR-1 by VEGF in cells lacking VEGFR-2 does not induce cell proliferation (32, 165). However, activation of VEGFR-1 by VEGF does induce cell migration, a response that is also induced as a result of VEGFR-2 activation by VEGF (31, 166, 167). These results indicate that the signal transduction cascades induced by VEGFR-1 and VEGFR-2 are somewhat different. The information regarding the signaling cascades induced by each of these receptors is limited, and it is not yet completely clear why VEGFR-1 does not induce cell proliferation in response to VEGF and VEGFR-2 does. VEGF promoted the binding and phosphorylation of the Shc and Nyc adapters, Grb-2 binding, and MAP kinase activation in porcine aortic endothelial cells expressing recombinant VEGFR-2. In contrast, MAP kinase was not activated by VEGF in cells expressing recombinant VEGFR-1 in two separate studies (32, 168). It is therefore possible that VEGFR-1 does not induce cell proliferation, because it does not activate MAP kinase. The SHP-1 and SHP-2 SH2 protein-tyrosine phosphatases physically associate with VEGFR-2 after stimulation with VEGF, raising the interesting possibility that both molecules participate in the modulation of VEGF-induced signals (168). Eventually, activation of the VEGF receptors results in the generation of proteases that are required for the breakdown of the basement membrane of blood ves-

sels in the first steps of angiogenesis (169–171), in the expression of specific integrins required for angiogenesis (172), and, finally, in the initiation of cell proliferation and cell migration.

The VEGF₁₆₅-specific receptor neuropilin-1

Endothelial cells also contain VEGF receptors possessing a lower mass than either VEGFR-2 or VEGFR-1 (133). It was subsequently found that these smaller VEGF receptors of the endothelial cells are isoform specific and bind to VEGF₁₆₅, but not to VEGF₁₂₁. It was therefore recognized that these receptors are not related to the VEGFR-1 or VEGFR-2 receptors that bind to both VEGF isoforms (87). It was previously observed that several types of nonendothelial cells express these VEGF₁₆₅-specific receptors (133), and an additional search revealed several types of prostate and breast cancer cell lines that express unusually large amounts of these isoform-specific receptors (173). The binding of VEGF₁₆₅ to these receptors is apparently mediated by amino acids residing at the carboxyl-terminal part of the exon 7-encoded peptide of VEGF₁₆₅ (173, 174). The genes encoding these receptors were identified by using a combination of VEGF₁₆₅ affinity chromatography and expression cloning. The affinity purification approach revealed that the receptors seen in breast cancer MDA-MB-231 cells are encoded by neuropilin-1 (167), a receptor previously identified as a receptor for several types of semaphorins. Semaphorins were initially characterized as factors that act as repellents of nerve growth cones (175, 176). In addition, the expression cloning approach has led to the identification of another VEGF₁₆₅ receptor, which turned out to be the product of the closely related gene, neuropilin-2 (167, 175). It was recently observed that neuropilin-1 also functions as a receptor for the heparin binding form of PlGF, PlGF-2, but not for PlGF-1 (177).

The neuropilins have a short intracellular domain (Fig. 1) and are therefore unlikely to function as independent receptors. Indeed, no responses to VEGF₁₆₅ were observed when cells expressing neuropilin-1 but no other VEGF receptors were stimulated with VEGF₁₆₅ (167). Nevertheless, gene disruption studies indicate that neuropilin-1 is probably an important regulator of blood vessel development as mouse embryos lacking a functional neuropilin-1 gene die because their cardiovascular system fails to develop properly (178). It is therefore likely that neuropilin-1 is a VEGF₁₆₅ coreceptor. This assumption is supported by experiments showing that VEGFR-2 binds VEGF₁₆₅ more efficiently in cells expressing neuropilin-1, and that this potentiating effect is subsequently translated into a better migratory response to VEGF₁₆₅ as compared to the migratory response of cells expressing VEGFR-2 but no neuropilin-1 (167). In contrast, PlGF-1 and PlGF-2 potentiated the mi-

gration of endothelial cells equally well. This observation may indicate that neuropilin-1 is not able to function as a VEGFR-1 coreceptor, since PlGF was reported to induce cell migration via activation of the VEGFR-1 receptor (177, 179).

The identification of neuropilin-1 as a coreceptor of VEGFR-2 may explain why the cardiovascular system of neuropilin-1 $-/-$ embryos fails to develop normally (178). The identification of neuropilin-1 as a coreceptor of VEGFR-2 may provide an alternative explanation as to why VEGF₁₂₁, a VEGF splice variant that does not bind to neuropilin-1, seems to be a less active mitogen than VEGF₁₆₅. However, it should be pointed out that the measured differences between the mitogenic activities of VEGF₁₂₁ and VEGF₁₆₅ are divergent in different studies because of unknown reasons (119, 136). The identification of neuropilins as VEGF₁₆₅ receptors also suggests that the different semaphorins may play a role in angiogenesis by interacting with neuropilins expressed in endothelial cells and, conversely, that VEGF₁₆₅ may activate neuropilin receptors of neuronal cells. Additional isoform-specific VEGF receptors may also exist, as suggested by a study in which a different VEGF₁₆₅-specific receptor was described (180). However, the gene encoding this receptor has not yet been identified.

CONCLUSIONS AND FUTURE DIRECTIONS

The last 3 years have witnessed an ever-growing interest in VEGF that has resulted in the publication of more than 1100 research papers. It was therefore impossible to cite all the relevant publications. In many instances there were several publications from different laboratories reporting similar findings. In such cases, due to space constraints, only one publication was cited.

The research conducted after the discovery of VEGF revealed that VEGF is a central regulator of angiogenesis and vasculogenesis. Several diseases, such as cancer, are characterized by abnormal angiogenesis, and it was realized that in many cases these diseases are accompanied by the aberrant production of VEGF. Moreover, it was realized that modulation of VEGF function may contribute to a successful therapeutic treatment of these diseases. However, at the moment VEGF signal transduction is not sufficiently understood. It is therefore likely that the study of VEGF signal transduction will be an area of intensive investigation in the near future. Research aimed at the elucidation of the mechanisms that fine-tune VEGF production is also likely to be at the center of attention. The understanding of these processes is essential for the successful development of methods aimed at the modulation of VEGF-induced angiogenesis.

VEGF and the VEGF family members represent but one set of proteins of a whole set of regulatory factors acting in concert to shape networks of mature, functional blood vessels (181). There are indications that different angiogenic factors such as bFGF and VEGF may promote angiogenesis by using subtly different mechanisms (172). It is thus reasonable to assume that a major research effort will be concentrated in the future on the mechanisms by which the activity of different angiogenic and anti-angiogenic factors is coordinated. These future research efforts should lead to a better understanding of angiogenesis and, consequently, to the development of better drugs aimed at the treatment of diseases associated with angiogenic disorders. [F]

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Angiogenesis,
Extracellular matrix,
Vascular Endothelial
Growth Factor,
Receptors, Pathology



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Attachment B

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SIGNAL RELAYS IN THE VEGF SYSTEM

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1. ABSTRACT

A considerable progress has been made during the past years in elucidating the molecular actors of angiogenesis. Vascular endothelial growth factor turned out to represent the major inducer of angiogenesis. Optional splicing of its pre messenger RNA generates various isoforms which differ not only by their storage in the extracellular matrix but also by their signaling pathways. VEGF binds and activates two tyrosine kinase receptors called VEGFR1 and VEGFR2 and neuropilin-1. The elucidation of the transduction pathways of each receptor suggests that VEGFR1 mediates cell migration whereas VEGFR2 mediates cell proliferation. The construction of internal images of VEGF by the anti-idiotypic antibody strategy allowed us to determine that quiescent endothelial cells need to be activated by so far unknown factors to become competent to respond to mitogenic signals and acquire an angiogenic phenotype. The discovery of the mechanisms of action of the VEGF system has allowed the design of promising drugs which already entered the pre-clinical or clinical assays.

2. INTRODUCTION

In adult mammals the vasculature is quiescent except during the physiological cycles of reproduction. Endothelial cells are among those exhibiting the lowest replication level in the body with only 0.01% cells engaged in cell division at any time. Additional requirements in oxygen or nutrients, as in the course of tumor progression, diabetic retinopathy or rheumatoid arthritis, result in the sprouting of new capillaries from pre-existing vessels, or angiogenesis. This local hypervascularization is thought to result from the release of soluble mediators by the involved tissues, which induce the switch of the quiescent endothelial cell phenotype to an activated one, so that the endothelial cells are then able to respond to mitogenic signals. The release of mitogenic growth factors switches the activated phenotype to an angiogenic phenotype. The interactions of angiogenic growth factors with their receptors provide the signals for cell migration, proliferation and differentiation to form new capillaries.

It was postulated half a century ago that hypoxia preceded the retinal neovascularizations observed in diabetic retinopathy or retinopathy of the premature (1). The hypothesis that tumoral progression was dependent on angiogenesis also led to the concept of "tumor angiogenic growth factors" (2). The search for hypoxic retina and tumor angiogenic factors proved misleading until purification and cloning of the genes encoding angiogenic growth factors was achieved. During the past decade it has become clearer that the angiogenic growth factors which are up-regulated during pathological angiogenesis are similar to those promoting physiological angiogenesis or vasculogenesis during embryogenesis. Several candidates have been identified such as acidic fibroblast growth factor, basic fibroblast growth factor, transforming growth factor α , hepatocyte growth factor and interleukin 8, all of which are able to induce neovascularization in avascular organs such as the cornea and mimic *in vitro* all the steps of the angiogenic cascade (induction of proteases, cleavage of the extracellular matrix, proliferation, migration and differentiation into capillary-like tubes). It is now evident that the biological factor purified in 1989, and designated vascular endothelial growth factor (3), vascular permeability factor (4) or vasculotropin (5) represents a key regulator of angiogenesis.

3. MOLECULAR BIOLOGY OF THE VEGF SYSTEM

3.1. VEGF ligands

Vascular endothelial growth factor (VEGF) has been purified from the conditioned medium of a variety of cell types including bovine folliculostellate cells (3;6), tumoral cell line AtT-20 derived from mouse anterior pituitary (5), guinea pig tumor (4), and a rat glioma cell line (7). This growth factor, also known as vasculotropin (5), was primarily described as a specific mitogen for vascular endothelial cells derived from large or small vessels, regardless of their species origin. It is angiogenic *in vivo* in the chick chorioallantoic membrane assay (5) and the corneal pocket assay (8). VEGF is identical to the previously described vascular permeability factor (9; 4).

Molecular cloning of the cDNA showed that VEGF shares an overall homology

of 18% with the B chain of platelet-derived growth factor (10; 11; 12). The human VEGF gene is organized in eight exons separated by seven introns (13). Alternative splicing of a single gene transcription product can generate multiple species. The full transcript encodes a 189 amino acids isoform (V189). A longer molecular species, (V206), which contains an additional 17 codons following the 24 codons encoded by exon 6 appears to be expressed only in embryonal tissue (14). The transcript encoding the 165 amino acids form, deleted of exon 6, is expected to generate the 45 kDa peptide following signal peptide cleavage (V165). A fourth transcript, deleted of exon 7, encodes a 145 amino acids isoform (15). A shorter transcript, deleted of exons 6 and 7 encodes a 121 amino acids isoform which compared to V165 presents a deletion of 44 amino acids between positions 116 and 159.

Over the past few years four VEGF-related genes have been identified: VEGF-B (16; 17), VEGF-C (18), VEGF-D (19) or VEGF-related protein (VRP), and placenta growth factor (20). These four VEGF-related genes generate glycosylated dimers which are secreted after cleavage of their signal peptide. They contain the eight cysteine residues that are highly conserved within the VEGF and the PDGF family.

3.2. THE VEGF RECEPTORS

3.2.1. The VEGF binding sites

Two classes of high-affinity binding sites were initially described on microvascular derived endothelial cells, with dissociation constant (K_d) values of 2-5 pM and 50-100 pM, respectively (21; 22). Cross-linking experiments showed that the molecular mass of these cellular binding sites corresponds to 180-200 kDa. These two classes of binding sites have also been found in cells which are not of endothelial origin, namely retinal pigment endothelial cells (23), stromal cells cultured from neonatal hemangiomas (24) or hair dermal follicle cells (25). However other cells such as lens epithelial or corneal endothelial cells (26) bind VEGF on a single high affinity binding site with a K_d in the 10 pM range and a molecular mass of 120 kDa. These cells migrate or differentiate, but do not proliferate when VEGF is added. Recent reports have stated that other binding sites specific for the exon 7-containing VEGF isoforms exist on cancer cells (27; 28). The content or the structure of membrane heparansulfate proteoglycans is likely to modulate the affinity for VEGF. Although heparin increases the binding of iodinated VEGF to endothelial cells (29), it does not modulate the biological activity of VEGF (30). Ligand autoradiography studies of fetal or adult tissues depicted a specific binding of VEGF on endothelial cells regardless of their proliferation status (31; 32). The distribution of VEGF and its binding sites during cyclical growth of corpus luteum suggests that they are hormonally regulated (32; 33; 34).

3.2.2. The VEGF receptors genes

Two VEGF tyrosine kinase receptors have been identified. The *fms*-like-tyrosine kinase (35) Flt-1, also called VEGFR1, and the kinase domain region (36) KDR, also called VEGFR2, or its murine homologue fetal liver kinase-1, Flk-1 (37; 38).

Both of these tyrosine kinase receptors bind VEGF with high affinity. Both receptors have seven immunoglobulin-like domains in the extracellular domain, a single transmembrane region and a consensus tyrosine kinase sequence that is interrupted by a kinase insert domain (figure 1). The recent identification in the rat retina of a second VEGFR2 transcript truncated of the COOH-terminal half of the intracellular kinase domain and the carboxyl region has demonstrated that these sequences are not necessary for transphosphorylation and function (39). The two promoters of VEGFR1 and VEGFR2 contain a 5' flanking sequence essential for endothelial specific expression (40; 41). The VEGFR2 promoter is sufficient to induce a 10 fold enhancement in the expression of foreign genes in endothelial cells compared to fibroblasts (42).

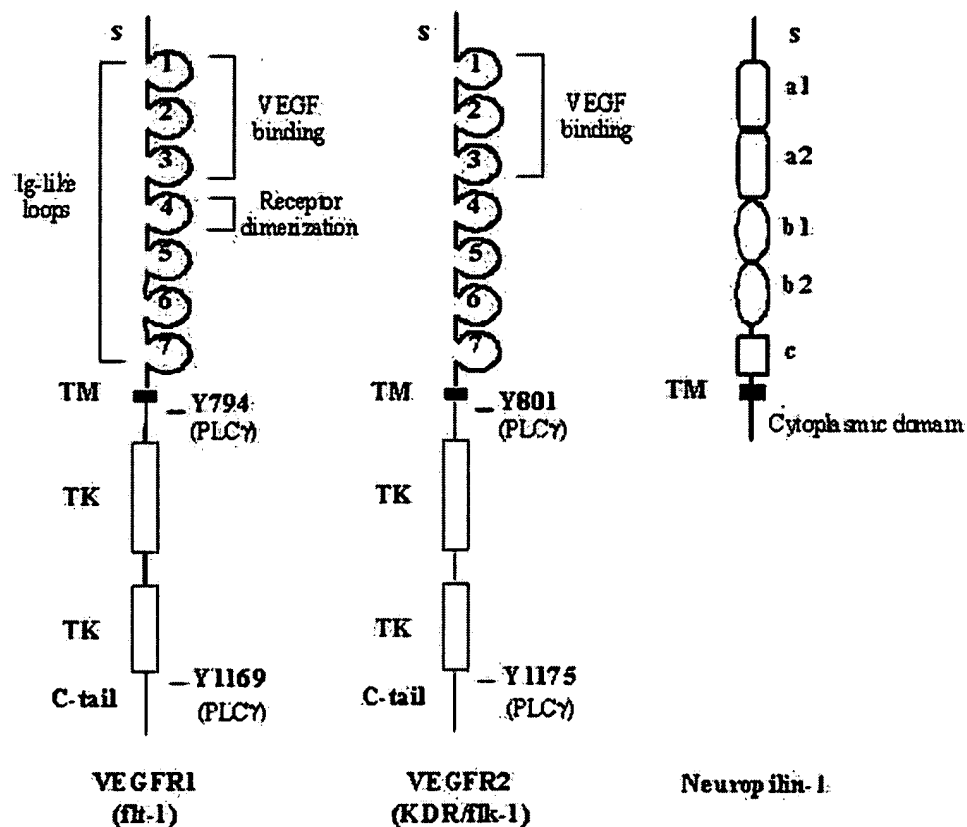


Figure 1: Molecular structure of the VEGF receptors. Diagram displaying the modular structure of VEGF receptors VEGFR1 and VEGFR2 (s), signal peptide ; (1-7) extracellular domain containing 7 immunoglobulin-like loops ; (TM) transmembrane domain ; (TK) tyrosine kinase domain. Neuropilin-1 (s), signal peptide ; (a1 and a2), complement C1r/s homology domain (CUB domain) ; (b1 and b2), regions of homology to coagulation factors V and VIII, MFGPs and the DDR tyrosine kinase ; (c), MAM domain.

VEGF binds to VEGFR1 with a K_d of 20 pM (35), whereas the K_d observed for PlGF1 or PlGF2 is significantly higher, in the 100-200 pM range (43). This receptor is expressed in cells which do not proliferate but migrate in response to VEGF, such as corneal endothelial cells or monocytes (44). Constitutive

expression of VEGFR1 allows cells to migrate in response to VEGF or PlGF (45). Lower affinity (K_d of 100-700 pM) has been reported for the binding of VEGF to VEGFR2 in transfected cells (46). However the affinity of dimeric receptors is 100 fold higher. Down regulation of VEGFR2 achieved by VEGFR2 antisense oligonucleotide transfection reduced the overall binding of iodinated VEGF to human umbilical vein endothelial cells or stromal cells derived from neonatal hemangiomas by only 10%, but this reduction appeared to concern a high ($K_d = 1$ pM) affinity binding site (24). VEGF-C, providing it is cleaved by proteases (47), binds and activates VEGFR2. Recently the HIV-1 transactivator Tat-1 has also been demonstrated as a VEGFR2 ligand inducing its phosphorylation (48). VEGFR2 constitutive expression allows cells to proliferate upon addition of VEGF (46).

A third member of this family of type III tyrosine kinases is represented by Flt-4, also called VEGFR3 (49; 50). Its expression is restricted in adults to lymphatic endothelial cells which suggests a role in lymphangiogenesis. It does not bind VEGF or PlGF but binds VEGF-C (47) and VEGF-D (51) in its native or cleaved forms.

Neuropilin-1, the previously identified neuronal cell guidance receptor of the semaphorin ligands (52), has been recently identified as a VEGF receptor (53). Neuropilin-1 modulates the interaction of VEGF with VEGFR2, and therefore the mitogenic activity of VEGF and it might also be involved in endothelial cell guidance (53) (figure 2).

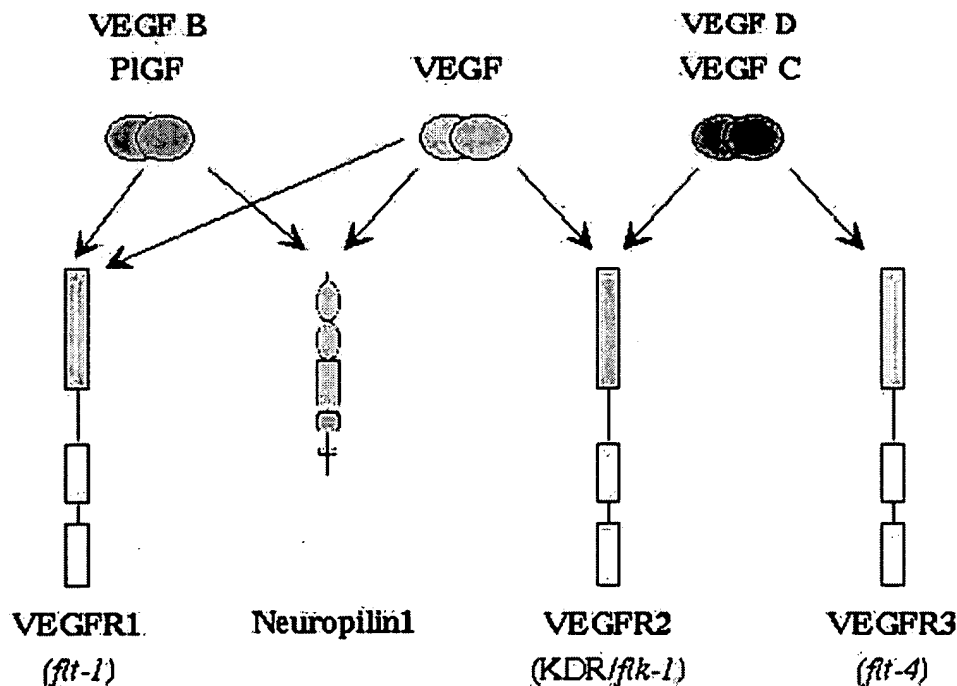


Figure 2: Interactions of the five VEGF ligands with the four VEGF receptors. Schematic representation of the distinct binding of the members of the VEGF family to the 3 tyrosine kinases receptors containing 7 immunoglobulin-like

loops. Splice variants which contain basic domains (VEGF 165 aa, VEGF 189 aa, PlGF 152 aa) bind also to Neuropilin-1.

3.2.3. Structural mapping of VEGF/VEGFRs interactions

The production of specific VEGFR2 agonists by anti-idiotypic strategy led to the hypothesis that critical VEGF determinants for each of its receptors should be distinct (54). Site-directed mutagenesis was used to localize the determinants that mediate VEGF binding to its two receptors (55). A model based on the crystal structure of PDGF-BB was used to perform alanine-scanning analysis. Although single mutations of Lys 82, Arg 84 and His 86 located in a hairpin loop displayed a moderate decrease in KDR binding, the triple mutation yielded to a strong inhibition of VEGF binding. Another study revealed that a second region (Ile 46, Ile 43) is critical for binding to the receptor and to a neutralizing monoclonal anti-VEGF antibody (56). Mapping of these determinants to the structure of the VEGF dimer shows that these two hot spots are located at each pole of the molecule. Similarly, negatively charged Asp 63, Glu 64 and Glu 67 are responsible for VEGFR1 binding. These mutants are still mitogenic whereas those which do not bind to VEGFR2 are not.

A third domain, encoded by exon 7 of VEGF is necessary and sufficient to allow the binding of VEGF to neuropilin-1 (53,57). This receptor also interacts with exon 6 of VEGF and PlGF1 (58).

The construction of various mutants of the Ig-like domains of each VEGF receptor has provided useful tools for analysis of their ligand binding domains. VEGFR1 mutants deleted of the second Ig-like domain fail to bind VEGF or PlGF, whereas the exchange of this domain with the second Ig-like domain of VEGFR2 restores VEGF binding similar to that observed in native VEGFR2, which means that this mutant no longer binds PlGF (59). However maximal VEGF binding to VEGFR1 only requires the presence of the 1-3 Ig-like domains (59) or only the domains 2 and 3 (60) whereas maximal transphosphorylation also requires the presence of the 4-7 Ig-like domains (61).

The crystal structure of VEGF has shown that its dimerization is similar to that of PDGF and symmetrical binding sites for VEGFR2 are located at each pole of the homodimer (56). Each site contains two functional hot spots composed of binding determinants presented across the subunit interface. The crystal structure of VEGF complexed with the second domain of VEGFR1 shows predominant hydrophobic interactions with the poles of the VEGF dimer. Five of the seven VEGF residues critical for tight binding to VEGFR2 are buried in the interface with the domain 2 of VEGFR1 in the complex (60).

3.2.4. Signal transduction

Although pioneer experiments using endothelial cells expressing each VEGF receptor constitutively led to the conclusion that VEGFR2 mediates cell proliferation, migration and actin reorganization whereas VEGFR1 does not signal for VEGF (46), it is now emphasized that VEGFR1 expression in naive cells mediates cell migration (44; 62).

Although both VEGF receptors are tyrosine phosphorylated (46; 64) *in vitro*, it seems that the tyrosine phosphorylation of VEGFR1 is not required for its function *in vivo*. The deletion of the tyrosine kinase domain of VEGFR1 does not impair blood vessel formation in homozygous mice (63). Several proteins are phosphorylated in endothelial cells (65; 66). Both receptors have the potential to signal through PLC γ (figure 3) via phosphotyrosine residues located in the juxtamembrane and carboxyl tail regions (67; 68). Although the use of the two hybrid system has demonstrated that VEGFR1 associates with PI3 kinase (69), this association has not been found in endothelial cells. Several adaptators of the Src family, such as Fyn and Yes are phosphorylated in response to VEGF/VEGFR1 but not to VEGF/VEGFR2 interactions (64). In contrast phosphorylated VEGFR2 associates with Shc, Grb2 and Nck, but also with the phosphatases SHP-1 and SHP-2 (70), activates the phosphorylation of the MAPK cascade (71) via the stimulation of Raf (72). VEGF also induces the tyrosine phosphorylation and recruitment of focal adhesion kinase and paxillin (73). Although the transduction mechanisms of the other ligands of the VEGF receptors remain unclear, it seems that tat-1 mimicks VEGF in inducing the phosphorylation of VEGFR2, MAPK and paxillin on Kaposi derived cells (74) (figure 3).

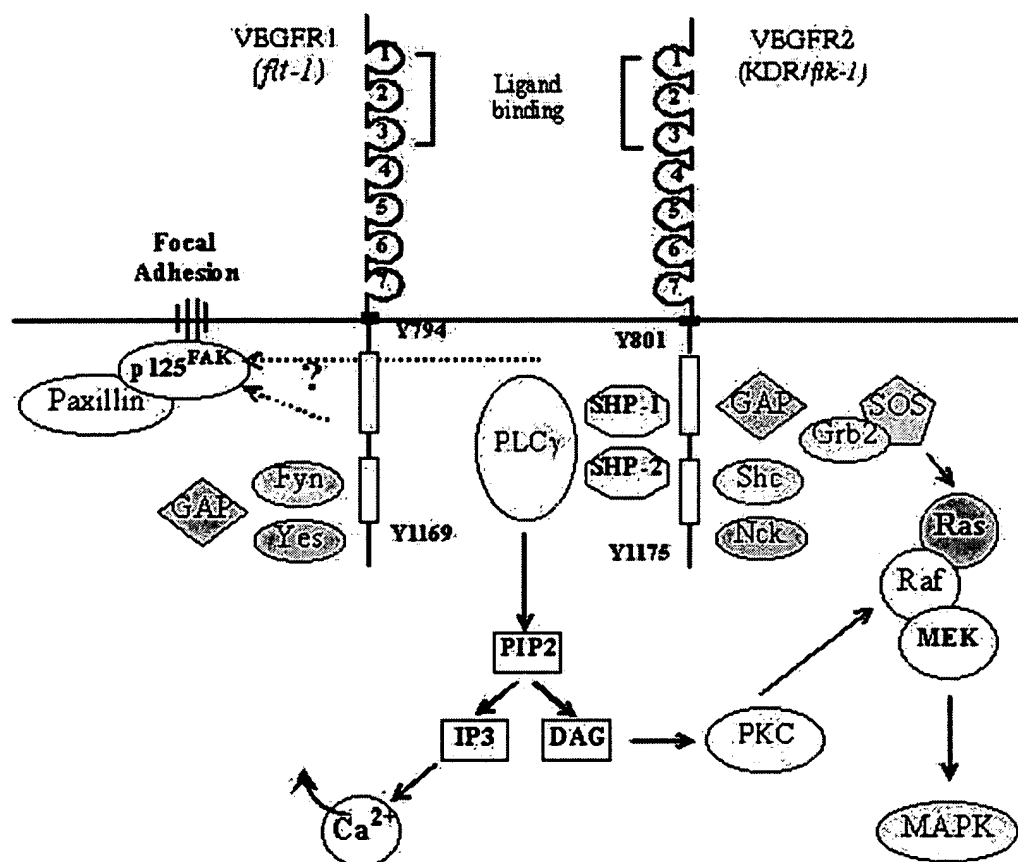


Figure 3: Signal transduction of the VEGF receptors. Schematic representation of the interactions of cellular adaptors to VEGF tyrosine kinases receptors.

3.2.5. Regulation of gene expression

It was postulated half a century ago that retinal neovascularization was a consequence of hypoxia, and that hypoxic retina contained angiogenic factors (1; 75). Similarly several tumors with extensive vascularization, such as multiform glioblastoma, exhibit hypoxic areas in the periphery of necrotic foci. These physiopathological observations led to a search for hypoxia-regulated angiogenic factors. Low oxygen tension dramatically up-regulates VEGF expression in tumoral cell lines (76; 77). However it is not clear whether hypoxia regulates VEGF receptors. It seems that VEGFR1 is upregulated whereas the expression of VEGFR2 is not (78; 79). Hypoxia induces the expression of VEGF in human endothelial cells which in turn activates VEGFR2 phosphorylation and cell proliferation (80). The up-regulation of VEGFR2 observed in endothelial cells surrounding hypoxic tissues seems to require the release of a yet unidentified paracrine factor (81).

The up-regulation of VEGF that occurs in virtually any tumoral cell line has led to many studies aimed to decipher the mechanism of VEGF overexpression during the switch of a normal cell to a transformed one. Although both VEGF receptors are expressed in normal adult endothelial cells (82, 83), their proliferation rate is very low. This is yet to be explained. However VEGF overexpression might in turn activate the expression of VEGFR1 (79) and TNF α increase that of VEGFR2 and neuropilin 1 (84).

4. BIOLOGICAL FUNCTIONS MEDIATED BY EACH VEGF RECEPTOR

4.1. VEGF activity *in vitro*

The identification of VEGF transcripts in cultured vascular smooth muscle cells (13; 14) and the response of endothelial cells suggested that VEGF was a typical paracrine factor involved in proliferation and/or survival of the endothelium.

Although most endothelial cells do not express VEGF, the endothelial cells derived from brain and retina capillaries secrete and respond to exogenously added VEGF (85). This functional autocrine loop suggests that in neural tissues, VEGF might contribute to maintenance of the fenestration in the blood brain barrier (86). VEGF up-regulates the expression of plasminogen activator and plasminogen activator type I inhibitor (87), tissue factor (30), interstitial collagenase and promotes tube formation of endothelial cells cultured in three dimensional collagen gels (30; 88).

The pioneer observation by Dvorak's group that several tumoral cell lines increased microvessel permeability, leakage of plasma proteins and formation of a fibrin gel facilitating endothelial cell sprouting led to the discovery of the permeability action of VEGF (9). This effect is thought to constitute a crucial step in tumoral angiogenesis and wound healing. It seems to be more likely exerted through an increase of the transcellular pathway inducing the formation of vesiculo-vacuoles (89) rather than through an opening of tight junctions leading to an increase of paracellular permeability. Inhibition of MAP kinase pathway but not protein kinase C or PI3 kinase prevents the permeability action of VEGF.

Since adult endothelial cells express VEGFR1 (91, 92), it is tempting to speculate that VEGF may be involved in endothelial survival. Indeed recent reports demonstrate that VEGF delays the senescence of human dermal endothelial cells (93). A unique effect of VEGF has been observed on the deposition of a scaffolding allowing these cells to attach, proliferate and escape apoptosis (94). When endothelial cells are coated on gelatin gels, VEGF prevents cell apoptosis induced by TNF α (95) or ionizing radiations (96).

Further studies have demonstrated that the vascular endothelial cell is not the sole target of VEGF which is a true interleukin since it stimulates the proliferation of IL2 dependent lymphocytes (97) the colony formation of granulocyte-macrophage progenitor cells (98) and inhibits the differentiation of dendritic cells (99). VEGF is also an autocrine growth factor for retinal pigment epithelial cells (23), hair dermal papilla cells (25), and stromal cells cultured from neonatal hemangiomas (24). In addition, VEGF is chemotactic for monocytes (44), lens epithelial or corneal endothelial cells (26), as well as a differentiation factor for osteoblasts (100) and 3T3 cells (101).

A recent report focused attention on the ability of VEGF to induce cell transformation. Constitutive expression of VEGF in rat retinal pigment epithelial cells leads to cell transformation; which is dramatically increased by FGF2 (102). This transformation is linked to a major overexpression of FGFR1. However neutralizing antibodies directed against VEGF or FGF2 have no effect on FGFR1 expression or cell transformation, suggesting that the target site of VEGF is intracellular.

4.2. Developmental regulation of the VEGF receptors

VEGFR1 (91) and VEGFR2 (37; 38) are selectively expressed in embryonic endothelial cells, first in the yolk sac and intraembryonic mesoderm, later in angioblasts, endocardium and in small and large vessels. The observation that VEGFR2 expression decreases at the end of gestation, whereas VEGFR1 persists in adult quiescent endothelial cells led to the hypothesis that they might be involved in different functions: VEGFR2 mediating the vasculogenic and angiogenic activities and VEGFR1 mediating vessel survival.

This hypothesis was confirmed by gene knockout experiments demonstrating that both receptors were essential for development of the vasculature. Null embryos for VEGFR1 fail to organize normal vessel channels, although endothelial cells proliferate, and die between day 8.5 and 9.5 (103). Both endothelial and hematopoietic cells fail to develop in VEGFR2 null mutant embryos which die at the same stage from distinct malformations (104). Complementation experiments have shown that VEGF is required for endothelial differentiation (105), and the analysis of null VEGFR2 embryo stem cells has shown that endothelial cells fail to proliferate and migrate from the primitive streak to the yolk sac and to intraembryonic sites of early angiogenesis (106).

4.3. Physiological activation of VEGF receptors in normal adults

The persistence of VEGF and VEGF receptors in organs where angiogenesis does

not occur, such as brain choroid plexus and ventricular epithelium (107) or kidneys (82) has led to numerous hypothesis. The most widespread hypothesis speculates functions of permeability or survival. However long term treatment of mice with neutralizing antibodies reduces tumor growth but has no effect on kidney or brain ultrastructure (108). *In vivo* VEGF induces vasodilatation through an NO-dependent pathway leading to acute and severe hypotension. This effect is distinct from its angiogenic effect since VEGFR2 agonists mediate angiogenesis but not hypotension (109).

In situ hybridization demonstrates that VEGF expression is spatially and temporally expressed during the menstrual cycle in the ovaries and uterus (110). The concomitant expression of VEGF receptors suggested that the VEGF system plays a role in hormonally regulated angiogenesis. This hypothesis has been recently confirmed by the same group by the demonstration that the selective decrease of VEGF bioavailability impairs corpus luteum angiogenesis (111). Hair follicle growth also requires cyclical angiogenesis to initiate the proliferation and survival of the dermal papilla. Immunohistochemical studies have provided insight into the cyclical expression of VEGF in the hair follicle where this latter may in addition act as an autocrine factor for hair dermal papilla cells. This hypothesis is in good accordance with the report of a concomitant decrease in VEGF expression and the onset of alopecia (112).

4.4. Activation of VEGF receptors in experimental angiogenesis

Any experimental model of angiogenesis associates the local delivery of the putative angiogenic factor and an inflammation resulting from the surgical trauma necessary to insert the device (slow releasing pellet or producing cells) containing the factor. Therefore the straightforward demonstration that VEGF acts by activating a VEGF receptor and not by releasing other growth factors from the extracellular matrix raises several methodologic points. For the instance it has been shown that basic synthetic peptides corresponding to the VEGF exon 6 sequence are angiogenic through the release of FGF2 from the corneal stroma (45). Although progress has been made in this area, the large number of heparin binding growth factor receptors makes screening of the distinct functions mediated *in vivo* by each receptor a difficult task.

The expression of VEGF receptors in adult endothelial cells which do not proliferate remains puzzling and raises serious questions about the function of their translation products in quiescent endothelial cells. Although the delivery of growth factors is impaired by their inability to reach their targets as a result of being sequestered in the extracellular matrix, systemic injections of VEGF do not induce the proliferation of endothelial cells of large vessels unless they had been previously activated by a trauma such as artery ligation or angioplasty (113). Accordingly, the ability of VEGF to bind to the proteoglycans of the vascular wall prevents it from reaching tumors and modulating tumoral angiogenesis (114). It seems therefore that the target of VEGF is not the quiescent endothelial cell, but rather the endothelial cells activated by inflammation. However a prerequisite to demonstrate that a growth factor activates *in vivo* a receptor is the obtention of circulating agonists specific for this receptor. We constructed circulating agonists mimicking the distinct domains of

VEGF interactions with each receptor by the use of the anti-idiotypic strategy (108).

These internal images selective for VEGFR2 induced the tyrosine phosphorylation of a set of proteins similar to the one observed upon VEGF addition, and induced cell proliferation but not migration. These VEGFR2 agonists stimulated the outgrowth of capillaries from the limbal vessels in rabbit and rat corneal pocket assays, demonstrating that VEGFR2 homodimerization was sufficient to induce angiogenesis and was not species-dependent. Corneal angiogenesis can also be mediated by the homodimerization of VEGFR1 since PlGF1 or PlGF2 alone may elicit angiogenesis (115; our unpublished results). Histology of the corneas demonstrated that VEGF led to the formation of corneal edema surrounding the pellet whereas homodimerization of VEGFR2 did not. This edema resulted probably from an increase of VEGF-driven permeability which we have shown to be mediated by the activation of VEGFR1 in the Miles assay.

These anti-idiotypic antibodies induced corneal angiogenesis in the absence of a local delivery of growth factor, suggesting that in this animal model of controlled angiogenesis, the proliferating phenotype switch of the endothelial cells is not linked to the presence of angiogenic growth factors. It is tempting to speculate that the functional expression of VEGFR2 on limbal endothelial cells is triggered by corneal cytokines released following the local trauma occurring during the graft of the pellet. Despite the fact that VEGFR2 mRNA are expressed in adult glomeruli and retinal pigment epithelial cells, no microscopic modification could be detected in kidneys or retinas thus indicating that VEGFR2 gene translation products are not functional in healthy organs. VEGFR2 appears as a functional marker of the endothelial cells which have switched to the angiogenic phenotype occurring in controlled and uncontrolled angiogenesis.

4.5. VEGF receptor activation in tumoral angiogenesis

The demonstration that VEGF mRNA is expressed in cancer cells, whereas the VEGF protein is also accumulated in endothelial cells located in the vicinity of cancer cells (116) has paved the way to numerous studies confirming the essential role of VEGF in tumoral angiogenesis (117; 118). VEGF is usually overexpressed in the hypoxic periphery of necrotic areas, whereas VEGFR1 and VEGFR2 are overexpressed in contiguous endothelial cells. In accordance with the previous finding that VEGF stimulates the proliferation of IL2-dependent lymphocytes, VEGF is also expressed in tumor infiltrating lymphocytes (99). Counting endothelial cells has been proposed as an independent factor reflecting the metastatic potential of prostate and breast cancer (119). Indeed a good correlation between vascularity and VEGF expression has been found in tumor samples, and VEGF seems to represent a useful independent prognosis marker. VEGF immunoreactivity is increased in the serum of cancer patients (120) and its decrease might indicate an efficacy of the chemotherapy used (121). However more studies are required to evaluate the potential interest of blood VEGF measure as an indicator of cancer metastasis or treatment efficacy since VEGF is present in platelets and therefore its blood content might in part result from the release of the platelets during the formation of the blood clot.

5. POTENTIAL VEGF RECEPTORS BASED THERAPEUTIC AGENTS

The genetic stability of endothelial cells constitutes an advantage for drug targeting because it is unlikely that such cells will acquire mutations and become resistant. This concept was developed in 1971 by Judah Folkman (2) and recently has been confirmed by the same group (122). Nude mice bearing tumors were treated with conventional chemotherapy. After three cycles of treatment some clones escaped and the treatment became inefficient. Conversely nude mice treated with the potent anti-angiogenic agent endostatin responded to each cycle and even ceased to relapse after a various number of cycles. Several attempts to inhibit VEGF bioavailability have proved to be successful: monoclonal neutralizing antibodies (123), antisense oligonucleotides (124), soluble VEGFR1 fusion proteins (125) or expressed by adenovirus carrying the cDNA sequence (126), anti-VEGFR2 antibodies (127) or immunotoxins targeted on VEGFR2 (Sordello, unpublished results) have been shown to inhibit tumoral progression or retinal neovascularization, as well as metastasis (128). It seems that the immunoneutralization of VEGF decreases the permeability of tumoral vessels without affecting the maintenance of the normal vasculature. Phase I and II clinical trials of humanized anti-VEGF antibodies are currently in progress. A synthetic inhibitor of VEGFR2 phosphorylation which has been reported as not affecting the tyrosine phosphorylation of other tyrosine kinases receptors (SU 5416) is also in phase II clinical trials. Although the specificity for angiogenic endothelial cells of some potential anti-angiogenic compounds remains to be demonstrated, they could act at least in part through interfering with VEGF signaling. For instance the 16K prolactin fragment inhibits VEGF-dependent MAPK activation (71) and angiostatin the VEGF-dependent tyrosine phosphorylation of paxillin (129) (figure 4).

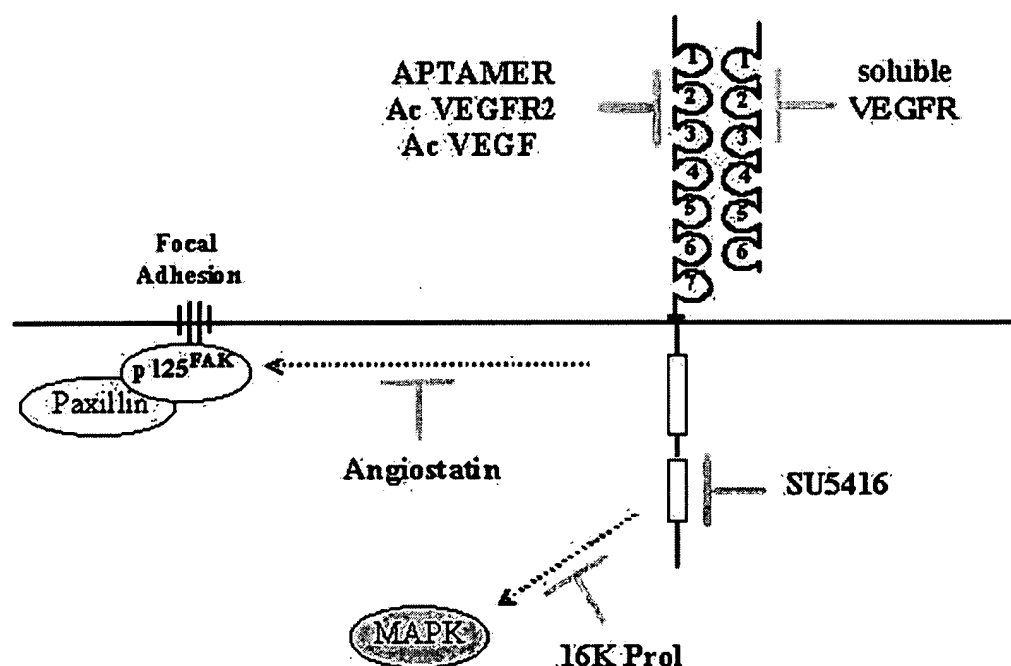


Figure 4: VEGF receptors based potential therapeutic agents. Representation of the molecular targets of potential anti-angiogenic agents interfering with VEGF

signal transduction.

The angiogenic activity of VEGF delivered either through protein infusion (113) or naked DNA intramuscular injection (130) seems to improve the recovery of limb or myocardial ischemia.

6. PERSPECTIVES

Experimental angiogenic assays have already ascribed an angiogenic activity to so many factors that it would be illusive to attempt to control angiogenesis by interfering with only one angiogenic pathway. However the growth factors tested in these assays act in an inflammatory context created by a surgical trauma which might overstate the actual role of a compound in pathological angiogenesis. It has been proved that several so-called angiogenic factors act by releasing endogenous angiogenic factors from macrophages or from the extracellular matrix. These assays therefore constitute a better reflection of controlled angiogenesis, as observed in wound healing or cyclical growth of the ovary, than the uncontrolled pathological angiogenesis observed in tumoral progression or diabetic retinopathy. Meanwhile the discovery of VEGF led to major insights in the comprehension of development of the vascular system as well as its role in physiological and pathological angiogenesis. Further studies using inducible gene knockouts of one or all the VEGF isoforms, and then tempting to recover the phenotype by activating VEGFRs signal transduction by injection of circulating receptors agonists such as anti-idiotypic antibodies should help to determine where and when the VEGF system is critical. The elucidation of the signal transduction properties of the tyrosine kinases VEGFR1, VEGFR2, VEGFR3 and their modulation by the recently identified neuropilin should also help to understand the mechanism of VEGFR2 silencing in quiescent endothelial cells or VEGFR2 transduction in angiogenic endothelial cells. Clinical trials of VEGF inhibition in cancers and VEGF infusion in myocardial ischaemia should also delineate the beneficial and adverse effects of each treatment.

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